Evaluation of Rice Varieties in *Drosophila melanogaster*

A THESIS SUBMITTED TO

THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY

FOR THE PARTIAL FULFILLMENT OF THE AWARD OF THE DEGREE OF
M.Sc. LIFE SCIENCES (AYURVEDA BIOLOGY)

BY

SRIVIDYA VATHSAL

UNDER THE GUIDANCE OF

DR. MEGHA
THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY
BENGALURU - 560064

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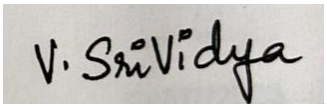
DECLARATION BY THE CANDIDATE

I declare that this thesis "**Evaluation of Rice Varieties in *Drosophila melanogaster***" submitted for the award of Master of Science to THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY, Bengaluru, is my original work, conducted under the supervision of Dr. Megha. I confirm that no part of the work reported herein has been submitted for a degree or examination at any other university. References, funding, and material obtained from other sources have been duly acknowledged, and no part of this dissertation has been plagiarised.



Place: Bangalore **Signature of the Candidate**

Date: 04-07-23

Name of candidate: Srividya Vathsal
Reg. No.: 2021MSCAB13

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CERTIFICATE FROM THESIS SUPERVISOR/S

This is to certify that the work incorporated in this thesis **"Evaluation of Rice Varieties in *Drosophila melanogaster*"** submitted by Srividya Vathsal was carried out under my supervision. No part of this thesis has been submitted for a degree or examination at any other university. References, help, and material obtained from other sources have been duly acknowledged. I confirm the originality of the work and that there is no plagiarism in any part of the thesis.

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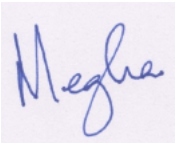
Megha, PhD Associate Professor, TDU	Supervisor	 04/07/2023

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Summary

There are many traditional rice varieties in the market that claim nutritional and health benefits. This work sought to understand the functional benefits of claims associated with Karungkuruvai, a black rice variety used in the Siddha system of medicine. First, we attempted to document the claims by holding discussions with stakeholders and reviewing available references in classical texts. Surprisingly, neither approach yielded a specific set of testable claims. Hence, available nutritional information on Karungkuruvai was deployed to assess how consuming this rice can impact physiology. *Drosophila melanogaster,* vinegar, or fruit fly was utilized as the animal model for experimentation. In flies, there is a strong co-relation between nutrition and life history traits. The quantity and quality of food provided impacts traits such as lifespan, resistance to stress, and fecundity. This study primarily employed fecundity (egg laying) as a readout for understanding the benefits of consuming Karungkuruvai (KK). Typical fly media is composed of corn flour, yeast and sugars. In our experiments, we compared how substitution of corn flour with Karungkuruvai or Ponni (a commonly consumed white rice variety) impacted fly fecundity. The experiments were conducted under two food regimens. Acute feeding where the rice diets were fed only for a short time as adults and continuous feeding where the flies were fed on these diets from egg to adult stages. In both regimens, KK fed flies displayed no difference in fecundity as compared to controls (corn flour). However, in acute feeding, flies fed on a Ponni diet exhibited 29% reduction in fecundity while on continuous feeding, they exhibited 48% reduction, as compared to controls. These differences were not due to feeding defects or differences in macronutrient compositions of the various flours. Surprisingly, 20d old flies exhibited a climbing deficit of ~ 56% on both rice diets. Further, we tested the effect of feeding the rice flours on early life malnourished flies. Remarkably, we observed that fecundity improved on a Ponni diet by 45% as compared to controls. Together, these suggest that *Drosophila melanogaster* can be used to study the functional benefits of rice varieties.

Personal Reflection

This project helped me understand how to approach a problem. This gave me first-hand experience to understand how difficult it is to get information from people on the ground. I had learnt how to initiate conversations in a structured manner and document them. I learnt that it was very important to define a question. Asking questions helped in giving clarity to the problem being addressed by breaking them into bits and pieces. Biological systems are different and not consistent all the time. There are lot of factors that can influence a result. A lot of planning needs to go into designing an experiment. It is worthwhile noting each step in the process. An experiment can fail, and mistakes can happen along the way. It's learning from them and moving on and not repeating them again. The importance of the reproducibility of experiments in science to be confident of the data generated.

In terms of skills, I learned *Drosophila melanogaster* husbandry*,* which is preparing media, flipping flies, sorting flies, anesthetizing flies using CO_2 and on ice, fecundity test which counting the number of eggs using Stereo microscope. I also learned to dissect the gut of the fruit fly. Learned to standardize assays, handle instruments, AOAC methods for proximate analysis, and presentation skills.

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1.0 INTRODUCTION

1.1 RICE

Rice (*Oryza sativa L*.) is the primary staple food for more than half the world's population.It is an extremely popular cereal widely consumed next to wheat. (Rathna Priya et al., 2019). Consumption and production of rice is remarkably high in the countries of Asia. It is also an integral part of their social rites, rituals, and festivals (Ahuja et al., 2008). India is one of the main countries for its production (Pushpam et al., 2019). Though India is self-sufficient in food production, its production between 1947 and 1960 was so bad that there were risks for the occurrence of famine. Therefore, the Green Revolution was initiated in the 1960s to increase food production, lower extreme poverty, and malnourishment in India and to feed millions (Eliazer Nelson et al., 2019).Green revolution that started in India in 1960s truly transformed agriculture, especially of wheat and rice. With the emphasis on monoculture and hybrid crops, the indigenous varieties of plants got ignored (Rathna Priya et al., 2019) (Ahuja et al., 2008).

1.1.1 RICE VARIETIES

Oryza sativa known as Asian rice was domesticated from the wild grass *Oryza rufipogon* or *Oryza nivara* about 10,000–14,000 years ago (Hour et al., 2020). Genetic evidence shows that the two main subspecies of rice – *indica* (prevalent in tropical regions) classified as long grain and *japonica* (prevalent in the subtropical and temperate regions of East Asia) classified either as medium grain or short grain come from a single domestication event that occurred 8,200–13,500 years ago in the Pearl River valley region of China. Another cultivated species, African rice, *Oryza glaberrima*, was domesticated in West Africa (“Rice Species,” n.d.; “Rice Types,” n.d.).There are more than 40,000 cultivated varieties of rice across the world . Rice has a rich genetic diversity, with thousands of varieties grown across the world. India is now a home to about 6000 varieties compared to 110,000 that existed earlier (Rathna Priya et al., 2019).

Different states in India grows different varieties of rice. Few mentioned below:

Rice Varieties	State
Basmati Rice, Sharbati Rice, Sugandha Rice	Punjab & Haryana
Sona Masoori, Prabhat, Godavari	Andhra & Telangana
Ratnachudi, Rajamudi, Mysore mallige	Karnataka
Nalihati,Tulasibasa,Pateni	Odisha
Matta, Ezhome rice, Pokkali rice	Kerala
Pankha Gura, Gopalbhog,Tulaipanji	West Bengal
Aduthurai, Ponni, Ponmani	Tamil Nadu

Table 1.1 Different Rice Varieties of Indian States

1.1.2 KARUNGKURUVAI AND PONNI RICE

Paddy, which is rice in the husk, comes in different colours while rice varieties are of different colours based on the processing type. Unpolished rice has the outer bran intact giving the rice brown, black or red colour. Polished rice is white which is obtained by the process of milling. Unpolished rice has higher nutrient content than milled and polished rice. Many factors influence nutritional value like quality of soil used in cultivation, kind of milling, method of preparation before consuming. The different varieties of coloured rice are considered for their health benefits (Rathna Priya et al., 2019).

Karungkuruvai Rice:-

One such variety of rice extensively used in Siddha medicine preparations is Karungkuruvai rice. The name is based on the colour of the husk and the season it grows. This rice has black husk and red kernels. This variety of rice matures in 3 months, in the Kuruvai season (June – August) and Navarai (December-March)(Chellakkan et al., 2016).

Ponni Rice:-

Ponni is one of the major high yielding white rice varieties of Tamil Nadu (Valarmathi et al., 2015). It was developed by Tamil Nadu Agricultural University in 1986 and is cultivated widely in various parts of India. 'Ponni' is a hybrid variety of 'Taichung65 and Myang Ebos 6080/2' (“Ponni Rice in Patent Row,” n.d.). “Ponni” in Tamil means “Gold”. Since Kaveri River is also

known as “Ponni”, this rice could have been named after that. It is usually cultivated on the banks of the river.

1.1.3 NUTRITIONAL QUALITIES OF KARUNGKURUVAI & PONNI RICE

Karungkuruvai Rice:-

The colour of the rice is because of the presence of Anthocyanin pigment which is an antioxidant. The phyto chemical analysis of the rice reported that the Karungkuruvai rice had a total phenol content of 220.11 ± 0.93 mg GAE/g and anthocyanin content of 52.54 ± 1.89 mg/L by-3 glc (3 glucoside cyanidin) (Devraj et al., 2020). About 24 volatile compounds have been identified in this rice. Curlone, a sesquiterpene found as a major constituent in turmeric and essential oils of turmeric is also found in this rice. Curlone is found to exhibit antioxidant and antitumor activity. The scavenging activity of Karungkuruvai rice was shown to be high (Krishnanunni et al., n.d.). The nutritive analysis of Karungkuruvai rice is as follows. (Balasubramanian et al., 2019).

Parameters/100g	**Karungkuruvai Rice (mg)**
Moisture	11.84
Total Ash	1.54
Total Protein	8.22
Crude Lipid	1.91
Dietary Fibre	1.92
Carbohydrate	74.57
Energy (Kcal)	348.35
Potassium	249.7
Iron	7.6
Calcium	37.7
Magnesium	94.7
Zinc	2.8
Phosphorous	344.5

Table 1.2 Nutritive Values of Karungkuruvai Rice

Ponni Rice :-

Ponni Rice is a widely cultivated rice in Tamil Nadu. It has been one of the successful hybridized rice varieties increasing the yield of rice. Ponni is known for its grain quality which is medium, slender, and white and its palatability (Agrobacterium Mediated Transformation White Ponni, n.d.).The nutritive values are as follows (Valarmathi et al., 2015)

Parameters/100g	**Ponni Rice (mg)**
Total Soluble Sugars	86±0.001
Total Ash	1.22±0.1
Total Protein NX5.95	8.7±0.01
Crude Lipid	2.42±0.09
Dietary Fibre	6.76±0.2
Total Starch	52.4±0.26
Total Amylose (%)	24.6±0.003
Potassium	237.3±2.08
Iron	1.3±0.003
Calcium	9.26±0.01
Magnesium	133±0.1
Zinc	1.22±0.02
Copper	1.23±0.03
Manganese	2.77±0.05
Sodium	22.6±0.005

Table 1.3 Nutritive Values of Ponni Rice

1.2 TRADITIONAL PERSPECTIVE

India has one of the richest traditions on medical knowledge with written texts of classical medicine and where knowledgeable practitioners are still using this information for treatment of

various health conditions. Knowledge of food and its properties have always been a part of traditional knowledge. Both in Ayurveda and Siddha systems of medicine, different varieties of traditional rice have its own uses (Balasubramanian et al., 2019).

1.2.1 VARIETIES OF RICE IN AYURVEDA

Ayurveda is an ancient medical system of India that is more than 5000 years old. It is considered to be a comprehensive integrative healing system. Ayurveda means “the science of life”. The texts in Ayurveda include recommendations for healthy diets, daily and seasonal routines and gives descriptions of herbs and herbal formulations for various health conditions (Arnold, 2022). Rice in the texts is classified in ”Sukadhanya”, grains with bristles. These are further classified as

- Sali, harvested in winter, cold in potency(sheeta virya) and sweet in taste and effect after digestion (madhura rasa and Vipaka). Raktasali, red variety is the best as it alleviates all the three vitiated doshas followed by mahasali and kalama.
- Sastika, harvested in summer, maturing in sixty days, cold in potency, light (laghu), sweet and alleviates all the three doshas. This also has minor varieties like varaka, uddalaka, cina, sarada, ujjala dardura, gandhana, kuruvinda.
- Vrihi, harvested in autumn, sweet in taste, sour effect after digestion (madhura rasa and amla vipaka), heavy(guru) and aggravates pitta. Patala variety aggravates all the three doshas (Agnivesa, n.d.)

Different rice varieties are given for different disease conditions and the effect of rice varies is based on how it is cultivated and stored. There are various medicinal properties to rice in the way it is processed. Rice grown in dry regions are light to digest while those grown in wet regions are heavy to digest. New rice is said to be an aphrodisiac while old, stored rice is light to digest. Puffed rice is light to digest while boiled rice stimulates digestive capacity. Thus, different varieties of rice have been used not only for regular consumption but also to manage different disease conditions and general well-being throughout the various seasons (Balasubramanian et al., 2019).

1.2.2. VARIETIES IN SIDDHA

Siddha is a traditional medicinal system in India which has a Tamil origin and is in Tamil language. This system uses metals and minerals to treat a variety of diseases and in this system, food is an ideal treatment (Chellakkan et al., 2016).

Classical Siddha texts contain about thirty traditional varieties whose names are based on season, origin, texture, colour, fragrance, size, duration, and specific location. Here is the list of the rice varieties: Kuruvai, Aanaikomban, Kadambu, Kalinga Samba, Kaar Kalundai, Katraazhai, Madangal, Manikathai, Manavaari, Milaghu Samba, Mosanam, Nariyan, Navarai, Neela Samba, Neervellai, Otadan, Pisaanam, Pisisni, Poombaalai, Kaadaikazhuthan, Sirumaniyan, Eesurakovai, Karunaivellai, Champa, Thillai, Thiruvaangam, Thuyyamalli, Vari Champa, Vaadai Champa, Vaalan, Vellaimilagu (Balasubramanian et al., 2019). Here are few specific descriptions from the list.

- Mai samba: This rice is like eyeliner that is dark black in colour.
- Malligai samba: This is pure white in colour and has a pleasant aroma like jasmine. The rice is also nourishing.
- Seeraga Samba: The grain resembles jeera seeds and is easy to digest.
- Kaivarai samba: The grain has linear marks like fingers. It nourishes the body and is healthy.
- Karungkuruvai: Karungkuruvai rice is highly recommended for use in regular diet. This helps in “pathiyam”, a restricted diet that helps the body to heal without medicines or to aid in better absorption of medicines. This rice is helpful for rheumatoid issues, joint and muscle pains of the lower back (T.V. Sambasivam Pillai, n.d.). Karungkuruvai Khadi is used in Siddha medicines for the elimination of heavy metals and for the enrichment of elements in components of Siddha drugs. These characteristics increase the curing properties and safety of the drugs for healing chronic diseases. Khadi is a fermented liquid, also known as “fermented rice vinegar,” which consists of various chemical compounds which is extracted from the boiled rice of Karungkuruvai (Chellakkan et al., 2016). This rice is useful in getting rid of poisons and skin diseases (The Research Institute of Siddhar’s signs, n.d.).

1.3 *DROSOPHILA MELANOGASTER* MODEL

Drosophila melanogaster, also known as fruit fly/ vinegar fly, has been widely used as a model organism. With a short life span of 60−80 days it is easier for life span studies. There are molecular similarities between humans and the fruit fly. This became known once the full genomes were sequenced for both. *Drosophila melanogaster* has about 14000 genes as compared to humans who have 21000 genes (By et al., 2018). About 50-60% of the fruit fly genes are orthologous to mammalian genes (Staats et al., 2018). Despite major anatomical differences, signaling pathways that regulate development, growth and metabolism are highly conserved between flies and mammals (Perrimon et al., 2016). Maintenance and reproduction of *Drosophila melanogaster* is simple and does not require complex equipment. Ethical issues are fewer in this research compared to studies in rats and mice. *Drosophila melanogaster* is now being used as a model organism in food and nutrition research (Staats et al., 2018).

1.3.1. LIFE CYCLE

Drosophila melanogaster male and female can be differentiated by the body size and colour of the abdomen. Females are bigger than males and the males have a black abdomen. The life cycle consists of 4 stages, egg/embryo, larvae, pupae, and adult. It is a holometabolous insect and complete metamorphosis is seen (Fernández-Moreno et al., 2007).

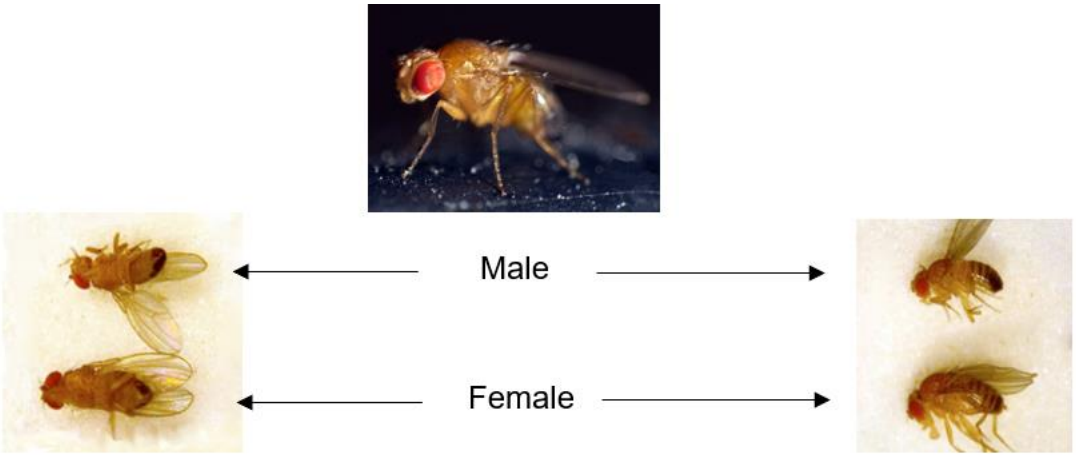

Figure 1: *Drosophila melanogaster;* differences between males and females
https://depts.washington.edu/cberglab/wordpress/outreach/an-introduction-to-fruit-flies/

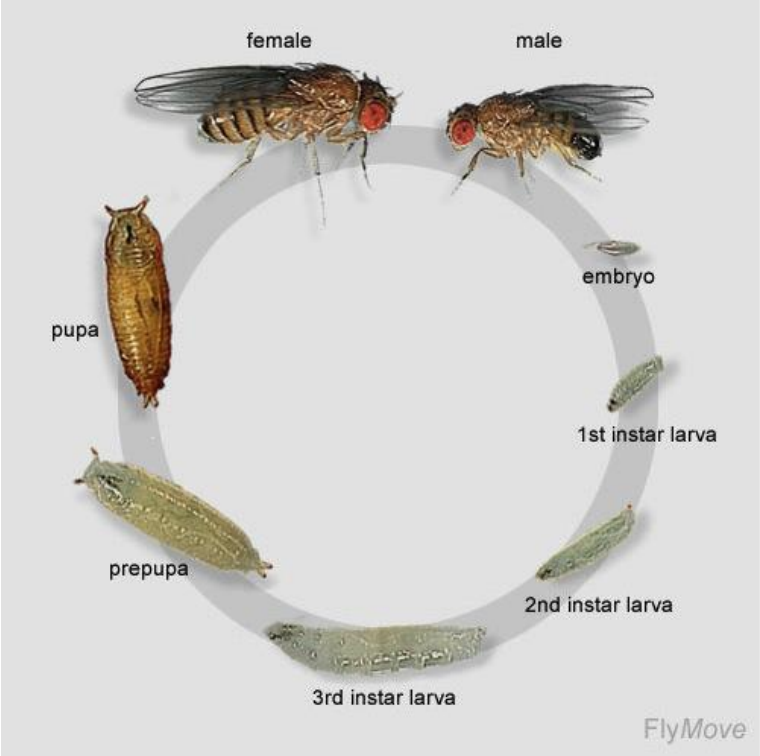

Figure 2: Life Cycle of *Drosophila melanogaster* at 25℃

https://depts.washington.edu/cberglab/wordpress/outreach/an-introduction-to-fruit-flies/

	Life Cycle at 25℃
Day 0	Female lays eggs
Day 1	Eggs hatch
Day 2	First instar (one day in length)
Day 3	Second instar (one day in length)
Day 5	Third and final instar (two days in length)
Day 7	Larvae begin to roam in this stage. Pupariation (pupal formation) occurs 120 hours after egg laying.
Day 10-12	Eclosion (adults emerge from the pupa case).

Table 1.4 Timelines in the lifecycle of *Drosophila melanogaster*

Females become sexually mature 8-10 hours after eclosion.

1.3.2 NUTRITION VS. EGG LAYING

The fruit fly model is emerging as a valuable model organism in food and nutrition research as there are several experimental methods, tools for analysis and genetic models available. Diet has an impact on many biological, physiological, and biochemical processes (Staats et al., 2018). A standard diet for *Drosophila melanogaster* is a complex medium comprising yeast, sugar and agar which can be easily and quickly prepared, providing all the necessary nutrients to flies. Important readouts in nutrition-based studies are measurements of food intake, body weight, composition as well as metabolic and digestive function. Other important are lifespan, fitness, locomotor activity, responses to stress in the environment and reproductive and developmental capacity. Fecundity is defined as the biological capacity for reproduction. It is determined via the assessment of egg laying in a particular time interval. Fertility is the proportion of living off-springs from laid eggs and involves monitoring the development and the observing the percentage of larvae, pupae, and adult flies (Eickelberg et al., 2022). Fecundity or egg laying is sensitive to nutritional availability and quality (Alves et al., n.d.). Reproduction is thought to be energetically costly and investment into reproduction is favored under optimal food conditions. Limitations in food might lead resources to survival rather than reproduction (Flatt, 2011). Macronutrient balance is an important determinant of fitness in *Drosophila melanogaster.* The concentrations of yeast and sugar have an impact on the lifespan and reproduction. Nutritionally the balance of protein carbohydrate ratio (P:C) is a critical determinant of the fitness. Flies fed with imbalanced content of protein and carbohydrate caused flies to age earlier and decrease egg laying. The eggs produced by females increase with increasing P:C ratio (Lee, 2015). The lifetime egg production was constant at higher protein intake P:C of 1:4. Further higher protein gradually decreases the egg production (Lee et al., 2008). It was found that flies reared on high carbohydrate diet (P:C 1:16) laid few eggs and visited high protein diets once introduced to them while flies reared on high protein diet (P:C 8:1) laid many eggs and took to high protein diets much later. Mated female flies show compensatory feeding which enables them to balance their protein and carbohydrates needs (Lihoreau et al., 2016).

2.0 MATERIALS & METHODS

2.1 TRADITIONAL CLAIMS

“Traditional” means as a part of a culture or long established. Karungkuruvai, known as a traditional rice, has very few references in Siddha literature. The cultivation of this rice meant that there could be some regular use of the rice that has been carried on from one generation to the other. There might be health and nutritional benefits for which this has been regularly used. To understand the practical use of rice and nutritional claims if any, discussions were held with people who have used this rice, grown or recommended this rice. Farmers, traditional healers, Siddha physicians mainly come under this category. Separate questionnaires were prepared for interactions with them. There were different sets of questions prepared. The following questions were asked of specific stakeholders; while the questions are documented here in English, based on the convenience of the person, they were administered alternatively in Tamizh.

For Farmers: -

1. Karungkuruvai Rice -What does the name mean? Why is it called so?
2. How do you farm it? With other crops? What is added to its growth? (Related to “Organic”, fertilizers etc.)
3. When is it usually sown and harvested?
4. Do you get the seeds for the next harvest or store them? How?
5. Do you eat this rice? Did you grow up eating this rice? How do you eat this rice? *(If yes, next Q)*
6. Which is the most preferred way of eating this rice? (Boiled/ Raw/ Polished/ unpolished/ Hand pounded)
7. Are you aware of any health benefits of this rice?
8. Any specific roga or disease condition that this helps in treating?
9. Anything else you like to share?

For Healers/ Physicians/ Others: -

1. Are you aware of Karungkuruvai Rice?

2. Do you recommend this rice to your patients? If yes, in which form (Boiled/ Raw/ Polished/ unpolished/ Hand pounded) and is there any specific brand or store you recommend? *(If yes, next Q, if no Q4)*
3. When do you recommend eating this rice? for healthy individuals & diseases (ask Diabetes, Elephantiasis if they don't mention. Orthopaedic /Arthritis, male fertility any connection to this rice variety that you are aware of?)
4. Do you think this rice has any health benefits? If yes, Like what? Have you or your patients personally experienced the benefits?
5. Is this rice mentioned in Siddha/Ayurveda/traditional texts? If yes, where have you come across this rice?
6. There are terms like Pooneeru/Muppu – Any idea on this?
7. I have heard that it might be helpful in Kayakalpam/Kayakarpam. Is that correct?

2.2 ASSAYS IN DROSOPHILA MELANOGASTER

A *Drosophila melanogaster* model was used to evaluate the rice diets (rice flours- Karungkuruvai and Ponni). Corn flour, which is the normal diet of *Drosophila melanogaster* was used as "control" and barley flour used as a non-rice control for the experiments because barley is highly recommended in Ayurveda. Food media was prepared to study egg laying in different diets.

2.2.1 RICE FLOUR –PREPARATION & CHARACTERIZATION

Materials Required:-

Karungkuruvai Rice(KK) and Ponni Rice (P) – 100g each

(Karungkuruvai boiled rice and Ponni boiled rice grains were given as a gift by Mr AV Balasubramanian, Director of Centre for Indian Knowledge systems (CIKS), Chennai; Batch number of KK and Ponni was 10/22-23)

- Weighing Scale
- DR MILLS DM-7412M Electric Dried Spice and Coffee Grinder
- Spatula
- Timer
- Electromagnetic sieves of Electrolab

- Ziplock bags

Method :-

- 100 g of KK and Ponni rice were weighed separately on a weighing scale in a zip lock bag and kept aside.
- Rice was filled till the max level indicated in the spice jar of the coffee grinder.
- The rice was pulse ground for 10 sec with a break of 5 sec. This process was continued for 5 minutes.This was mixed regularly with a spatula. Care was taken to ensure the jar did not heat up. A timer was used to monitor the duration .
- Once ground, the rice was transferred into the zip lock bags and labelled according to the rice ground.
- To characterize the size, individual rice flour was passed through electromagnetic sieves of Electro lab. The sieves used were ASTM 40 (425µm), ASTM70 (212µm), ASTM140 (106µm), ASTM200(75µm) sizes.
- The power setting was set to 20, time of 10 min and with continuous shaking .
- Most of the rice flour was collected in sieves 212µm and 106µm that was less than 425µm.
- Hence rice flour passed through sieve ASTM 40 (425µM) was used for experiments.

2.2.2 MEDIA PREPARATION

COMPOSITION OF MEDIA: 100 ML

	Normal Fly Media Corn Flour (Control)	**Barley Flour Media (BF)**	**Karungkuruvai Rice Flour Media (KK)**	**Ponni Rice Flour Media (Ponni)**
Flour	Corn Flour 8g	Barley Flour 8g	KK Rice Flour 8g	Ponni Rice Flour 8g
D-Glucose	4g	4g	4g	4g
Sucrose	2g	2g	2g	2g
Agar	0.8%	1%	1%	1%
Yeast Extract	5g	5g	5g	5g
Benzoic Acid	700µL	700µL	700µL	700µL
Orthophosphoric Acid	600µL	600µL	600µL	600µL

Propionic Acid	400µL	400µL	400µL	400µL
Distilled water	100mL	100mL	100mL	100mL

Table 2.1 Media composition

Materials Required: -

Corn flour	Corn sourced locally from Yelahanka and ground into flour
Ponni Rice Flour & KK rice Flour	Ground in house as per 2.2.1
Barley Flour	Sourced from I-AIM Healthcare Center pharmacy, this is sold as Yava churna
D Glucose	Qualigens; Catalog number Q15405
Yeast Extract	HIMEDIA; Catalog number RM027
Sucrose	Qualigens; Catalog number Q15925
Agar	Qualigens; Catalog number Q21185
Benzoic Acid (2mg/mL) Stock	Prepared from Qualigens; Catalog number 211665
Orthophosporic acid (85%)	Qualigens; Catalog number Q29245
Propionic Acid	Qualigens; Catalog number Q26955

Table 2.2 Source of the media components

- Weighing scale
- Spatula
- 500mL Fischer glass beaker
- Measuring cylinder
- Distilled water
- 100 mL small beakers as containers to weigh

- IFB Microwave
- Autoclaved Bottles or vials
- Micropipette (100µL-1000µL) & tips
- Muslin cloth
- Cotton plugs
- Refrigerator

Method: -

- The flours, glucose, sucrose, agar and yeast extract were weighed in small beakers as per the measurements in the media composition table 2.1)
- Each media was prepared separately (Control, KK, Ponni, BF)
- The media contents from the small beaker were transferred to a 500mL Fisher beaker that could be used in a microwave. 100 mL of distilled water was added using a measuring cylinder.

Each diet was cooked in a microwave according to the time (in sec) mentioned in the table below.

Corn Flour (sec)	**Barley Flour (Sec)**	**KK Flour (Sec)**	**Ponni Flour (Sec)**
30X30x 10x10	30x30x10 x10	30x30x25x10x10x10x10x 10x10x10x10x12x12x15x15	30x30x25x11x11x 11x11x10

Table 2.3 Time in sec for media preparation in microwave for each media

The beaker was taken out and stirred using a spatula after each time in the microwave.

- Once the media thickened, the beaker was taken out of the microwave carefully, and allowed to cool. After the vapours subsided, benzoic acid, orthophosphoric acid and propionic acid were added as per the media composition table and mixed thoroughly.
- Prepared media was poured into autoclaved bottles without pouring towards the walls.
- The bottles were labelled as per the media added and covered using muslin cloth and left to cool for at least 2 hrs.
- Once cooled, the bottles were plugged with cotton and stored in 4°C in the refrigerator.100mL of media was enough for 8 bottles.

2.2.3 FECUNDITY OR EGG LAYING ASSAY

This experiment was spread over 9 days and was done for 2 experimental regimens.

- Short term feeding -Flies fed respective flours for 3 days as adults only after a time of maturation on normal media for 2 days post-eclosion. Development media – normal corn flour.
- Long term feeding -Flies fed respective flours throughout development i.e., from egg/ embryo, larval stages to adulthood.

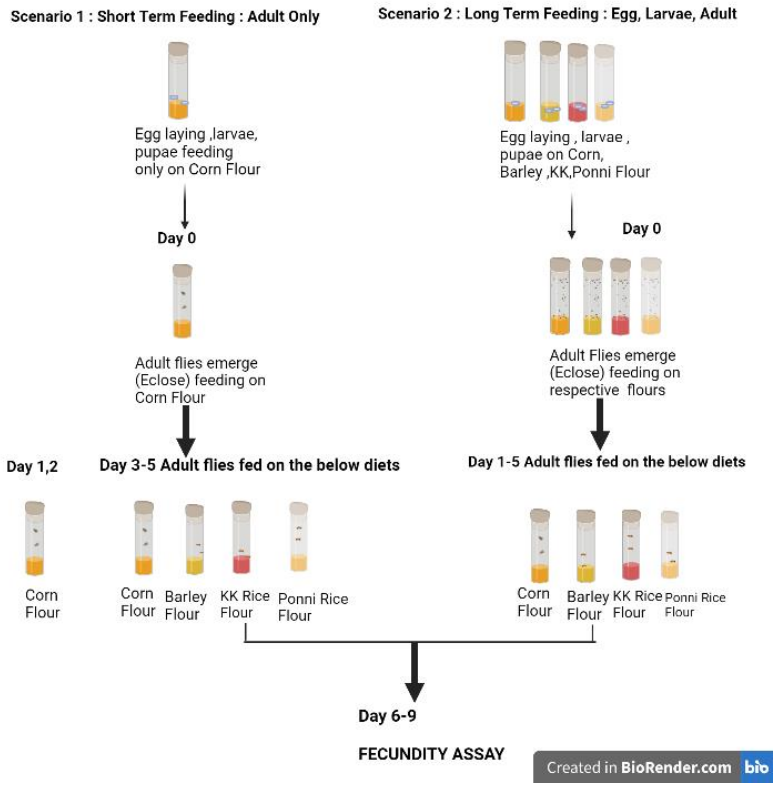

Figure 3: Pictorial representation of short term and long term feeding of fecundity assay. Note: Flies here refer to *Canton-S* wild type

Fecundity experiments:-

Fecundity experiments were done in cut bottles for the ease of counting the eggs. Before the experiment, the cut bottles were prepared with the respective media.

Materials:

- Materials required for media preparation as per the media composition table 2.1

- Cut bottles.
- Insulation tape
- Cotton plugs
- Click counter
- Refrigerator
- Stereo Microscope
- Incubator set at 25.2℃
- Ice
- Soft brush

Method:

- Media was prepared as mentioned in media preparation table 2.1.
- The cooked media was poured into cut bottles and once the media cooled, the upper part of the bottle was taped to the lower part using an insulation tape and cotton plugged. The experimental bottles were labelled clearly and stored in 4℃ in the refrigerator. 9 cut bottles were prepared for each media (Total of 36 cut bottles were prepared).
- From the flies fed as per the experimental regimen in figure 3 till day 5,on day six,18 females and 9 males were ice sorted for each media using a soft brush.
- The flies were transferred to 3 cut bottles with 6 female and 3 male flies in each, for every experimental media.The time of flipping was noted and the bottles were placed in the incubator.
- After 24 hrs, the cut bottles were taken out of the incubator and flies were flipped on to the next set of cut bottles. The eggs laid on day 6 were counted using a stereo microscope. Click counter was used to count the egg number .
- This was repeated 3 times for a total of 72 hrs.
- The eggs of each bottle were counted. Any deaths of female flies were also noted. Data was interpreted and results analyzed.

2.2.4 FEEDING ASSAY

2.2.4.1 FEEDING CUPS: -

Steps:-

Day 0	Flies eclosed from Corn
Days 1-3	Males and females sorted & flipped on corn vials for 3 days.
Days 4-5	Flipped on 1% agar vials with corn cups for 2 days.
Days 6,7,8	Flipped on 1% agar vials with Ponni and Corn cups respectively; 1% agar vials with evaporation cups without flies were also kept for 3 days
Days 6,7,8	Weight of cups checked cups before and after 24 hr. feeding period for both diets.

Table 2.4 Flow of the feeding assay

Materials:

- Materials required for media preparation of Corn flour and Ponni rice flour as per the media composition table.
- 1% Agar Vials
- Food Cups (lid of PCR tubes 4mm in diameter)
- Double sided tape
- Analytical weighing balance
- Forceps
- Micropipette & tips
- 1mL microcentrifuge tubes
- Cotton plugs
- Petri plates
- Dry heat block

- Incubator set at 25.2℃.

Method:

- 1% agar vials freshly prepared on days 4-8.
- 10 mL of media was prepared for food cups (Corn & Ponni) calculated according to the media preparation table. The cooked media for Corn and Ponni was poured into 1 mL microcentrifuge tubes and stored in 4℃.
- PCR tube lids were cut and kept ready for the food to be poured. The cups were placed on petri plates marked for the respective media for ease of use.
- The microcentrifuge tubes were placed in dry heating block set at 99.6℃.
- The melted media was pipetted into the food cups and allowed to cool.
- The 1% agar tubes were checked and ensured there was no water in them.
- 3 food cups for males and 3 for females per media were prepared and 3 evaporation cups for each media were also kept .
- The weight of the cups with the media was taken in an analytical balance before sticking the cups on to the 1% agar vials .The cups were stuck at a distance of 3mm from the agar.
- The vials were kept horizontally.
- On days 4 and 5, corn cups were placed in the agar vials and males and female flies were flipped on to separate vials. 6 vials with males and 6 vials with females and 6 vials with evaporation cups were kept. About 20 male flies and 20 female flies were flipped into the vials. The vials were kept horizontally in the incubator .
- On days 6, 7, 8, Ponni cups were introduced and hence 9 vials (3 for males, 3 for females, 3 with evaporation cups) for Ponni and similar for corn were prepared. Flies flipped into new vials for 3 days.
- The weights of the food cups were noted before and after 24hr feeding.
- Results were analyzed.

2.2.4.2 Erioglaucine Dye method

Quantification of the food eaten by the flies were done by this assay. Spectrophotometer was used measure the absorbance of the coloured lysate. The standard curve was prepared from the known concentrations of the dye and concentration determined.

Materials:

- Ponni and Corn media bottles
- Ponni and Corn media bottle with Erioglaucine dye(0.5%)(Sigma-Aldrich Erioglaucine disodium salt- Catalog Number 861146-25G)
- Incubator
- Ice
- Soft brushes
- Dissection forceps
- Stereo Microscope
- Microcentrifuge tubes
- Homogeniser
- Centrifuge
- Micropipettes and tips
- 96 well plates
- Spectrophotometer
- -20℃ Refrigerator

Method:

- Ponni and Corn media bottles were prepared as per the media composition table.
- For the bottles with 0.5% Erioglaucine dye, the dye to be added was calculated from the 25% stock that was available. For 30mL of media, 600µL of the stock was added and mixed and poured into bottles accordingly.
- Flies that eclosed were flipped on corn and Ponni media respectively and placed in the incubator. This was repeated everyday till day 4.
- On day 5, the flies were flipped on to the media bottles with 0.5% Erioglaucine dye and were incubated for 12-16 hr. The flies fed on the media with the dye.

- Few flies were left to feed on the media bottles without the dye.
- After 16 hrs in the dye, the flies were flipped on to corn and Ponni media respectively for 30min.
- The coloured and normal flies were ice sorted using soft brush; 5 males and 5 females each (with dye and without) were taken in 3 microcentrifuge tubes accordingly.
- The flies were killed by keeping at -20℃ for 2hrs.
- After 2 hrs, the head, wings, and legs were removed under the stereo microscope with dissection forceps and only the thorax and abdomen of each fly was taken. 150µL of distilled water was added to each of the microcentrifuges that had 5 flies.
- The abdomen and thorax of flies were crushed using a homogeniser.
- The microcentrifuge tubes were centrifuged at 14000rpm for 10 min at room temperature.
- 120µL of the lysate/ supernatant was taken. This was centrifuged again at 14000rpm for 10 min at room temperature.
- 100µL from this lysate was taken and loaded on a 96 well plate. This was done for all microcentrifuge tubes of Ponni and Corn and flies with dye and without.
- Along with the lysate, six known concentrations of dye, 1.25ng, 2.5ng, 5ng, 10ng, 20ng were also loaded.
- The absorbance was measured at 630nm.
- The standard curve was prepared, and concentrations calculated.

2.2.5 CLIMBING ASSAY

Assay done on Day 20 flies (Long term fed) to check the aging based on different diets. Males flies were taken for the assay.

Materials :-

- 50mL clean glass cylinder with an 8cm marking
- Soft brush
- Ice
- Empty vials

- Cotton plug
- Fly pad
- Timer

Method:

- 2 vials of male flies were collected by ice sorting (20 flies per vial) of each of the media respectively.
- The flies were incubated in the respective media vials for 30min.
- Flies transferred into empty vials and cotton plugged.
- The flies from the vial were transferred into the cylinder and plugged with cotton.
- The flies were allowed to rest for 1-2min.
- The bottom of the cylinder was tapped on the fly pad and the timer was set to 12sec.
- The number of flies that crossed the 8 cm mark was counted.
- The procedure was repeated 3 times for each vial with a gap of 2min.
- From this data, the average percentage of flies that crossed the marked line was calculated.

2.2.6 EGG LAYING IN CONTROL AND EARLY LIFE STARVED (ELS) FLIES FED ON RICE DIETS.

Control and ELS flies are given water stress during the third instar larval stage.

- Control flies: Post stress, flies developed from larvae transferred to normal diet.
- ELS flies: Post stress, flies developed from larvae transferred to 100mM sucrose media.

The experiment was spread over 9 days with the following experimental regimen. From day 0, both control and ELS flies were fed on corn, KK rice, Ponni rice and barley till day 5. The fecundity assay done from day 6-9 was done as per 2.2.3

3.0 RESULTS & DISCUSSION

3.1 TRADITIONAL CLAIMS

The network of traditional healers, farmers, and physicians available through TDU campus faculty, was used to identify stakeholders. Phone conversations were undertaken based on the format indicated in Materials and Methods.

Profession	Location	Summary of information received on karungkuruvai rice
Organic Farmer 1	Cuddalore	-Grown in the kuruvai season -No fertilisers required to grow -Awareness of traditional rice more after COVID -Traditional rice is used for hormone imbalance issues.
Organic Farmer 2	Cuddalore	Regularly consumes the rice as grown in fields. The rice is naturally resistant to many plant diseases.
Folk Healer	Cuddalore	Helps in treating “Kushta roga”, flattened rice is used to treat white patches with 60% cure, this rice increases the immunity overall .
Siddha physician 1	Kerala	Have never heard of this rice variety
Siddha physician 2	Chennai	Used in “Annakadi”, purification of medicines of Siddha drugs
Siddha physician 3	Thanjavur	The rice is grown in Thanjavur but not easily available for consumption .
Siddha physician 4	Bangalore	Heard of the rice but never used .
Siddha physician 5	Bangalore	Heard only in “Annakadi” preparations
Siddha physician 6	Thanjavur	Probably used for skin diseases , renal diseases to remove excess salt as per some books available

Siddha physician 7	Chennai	Don’t recommend to patients as nutritional benefits not known, The way it is grown is traditional with no pesticides and tends to retain its “innate genetical values.”

Table 3.1 Summary of information received on Karungkuruvai rice

Surprisingly, Siddha physicians who were contacted for this study were unable to provide a reference in their classical texts for the use of this rice. Furthermore, from a traditional perspective there is not much awareness on the rice or it's nutritional claims. It appears that the appeal of the rice is due to its agricultural advantages- it is a disease resistant species and easy to grow. There are some books that were written by Siddhars that mention Karungkuruvai rice for skin and immunity related problems, but discussions undertaken for this thesis did not provide immediate references; indicating that to investigate traditional claims a wider net has to be cast and academically minded Siddha physicians with in-depth knowledge of their classical texts have to be approached. In conclusion, there was insufficient information to build experiments to specifically test traditional claims. Hence, we proceeded to understand nutritional benefits in the framework of lifehistroy traits.

Drosophila melanogaster is a very good model to understand nutritional impact on lifehistory traits. One of the traits that we had chosen was egglaying or fecundity. The macronutrient content has an impact on egg laying. Depending on the nutritient availability, energy tradeoffs happen and there is a reduction in egg laying or increase in life span .

Healthy ageing also depends on the diet . We had checked for this in the flies by doing the climbing assay .

3.2 CLIMBING ASSAY:

The below graph shows the percentage of flies that were able to cross the 8cm mark on day 20. The flies were continuously fed on the diets mentioned.

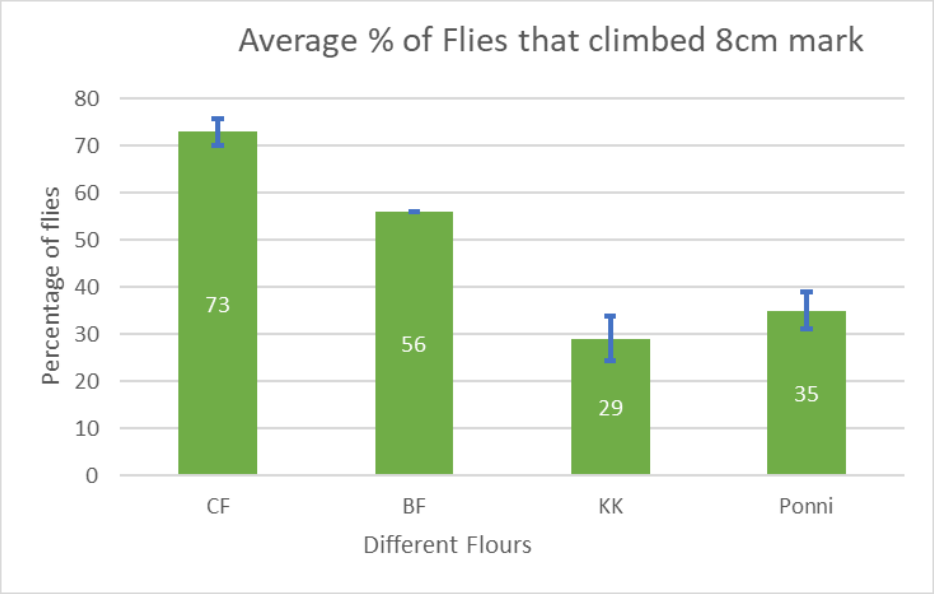

Figure 4: Average % of flies that climbed 8cm, CF- corn flour, BF- barley flour, KK-Karungkuruvai rice flour, Ponni- Ponni rice flour

From the graph the flies fed on corn flour and barley flour were better in crossing the 8cm mark. Rice diets were not very favorable for aging flies.

3.3 FECUNDITY ASSAY:

- In short term feeding, compared to control diet (corn), flies fed on Karungkuruvai diet showed a reduction of 14% in egg laying while flies fed on Ponni rice flour showed a reduction of 29%.

Short Term Feeding	**Average No of Eggs/female /day ± SD**
Control (Corn)	28±1.00
Barley	24±2.05
KK	24±1.94
Ponni	20±2.37

Table 3.2 Short term feeding:Average no. of eggs/ female/ day ±SD

- The graph represents the average number of eggs per female per day after short-term feeding on the respective diets.

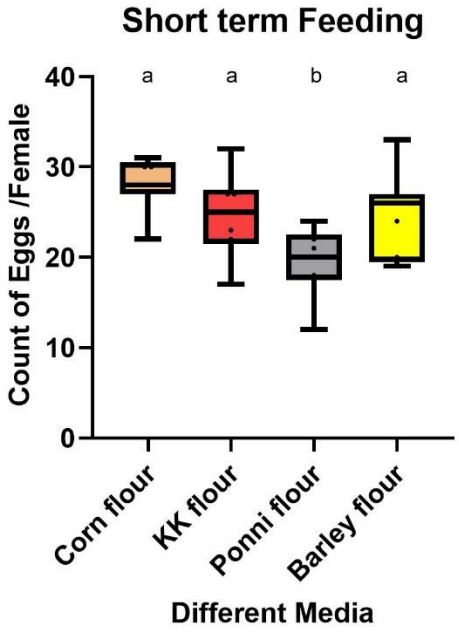

Figure 5: Short term fecundity plot where n=9 sets; each set has 6♀,3♂. One way ANOVA was performed. Letters above data indicate statistical differences between data sets by a post-hoc Tukey analysis.

- In long term feeding of diets, in comparison to control, Karungkuruvai diet showed increase by 26% while 48% decrease was seen in Ponni rice flour diet. There is an increased trend but it was not statistically significant for Karungkuruvai.

Long Term Feeding	**Average No of Eggs/female /day ±SD**
Control (Corn)	23±1.29
Barley	22±3.73
KK	29±3.03
Ponni	12±1.68

Table 3.3 Long term feeding:Average no. of eggs/ female/ day ±SD

- The below graph represents the average number of eggs per female per day after long-term feeding in the respective diets.

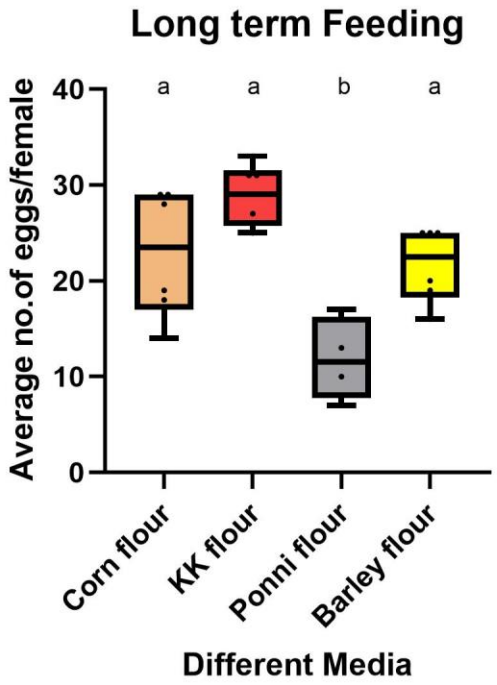

Figure 6: Long term fecundity plot where n=6 sets; each set has 6♀,3♂.One way ANOVA was performed. Letters above data indicate statistical differences between data sets by a post-hoc Tukey analysis.

From short term and long term fecundity experiments, there is a difference in the egg laying by flies on Ponni rice diet. This could be due to energy differences in the diet that the flies are fed on. Protein:carbohydrate ratio in diet is one of the key parameters for fecundity. Life time egg production was constant at a P:C ratio of 1:4. The below shows the proximate values of the flours sent for analysis to Eurofins company.

Parameters	Units	Corn Flour	Barley Flour	KK Flour	Ponni Flour
Moisture	g/100g	9.65	11.48	11.76	12.63
Total ash	g/100g	0.48	0.66	1.83	0.56
Total fat	g/100g	1.69	0.68	3.52	0.38
Energy (kcal)	kcal/100g	367.93	354.83	363.24	349.14
Total Carbohydrates	g/100g	80.16	80.3	74.3	78.9
Protein	g/100g	8.02	6.88	8.59	7.53
Iron	mg/100g	1.51	1.95	1.97	1.01

Table 3.4 Proximate Value table of flours sent to Eurofins

Based on the table above, total energy, protein, carbohydrate and iron content was calculated in the fly media that is used in experiments. Protein in yeast extract was also used in the calculation for protein content.

Media (100mL)	Corn Flour Media	Barley Flour Media	KK Flour Media	Ponni Flour Media
Total Energy (kcal)	55.62	55.19	55.86	54.73
Total Protein(g)	4.42	4.34	4.47	4.39
Total Carbohydrate (g)	13.51	13.68	13.20	13.57
Total Iron (mg)	0.42	0.45	0.45	0.38

Table 3.5 Calculated values in the media

Energy wise, there was no difference seen across the different diets. Protein:Carbohydrate is also constant at about 1:3. Another scenario for decrease in fecundity would be if the flies were not eating Ponni rice diet.

3.4.1 FEEDING ASSAY-FEEDING CUPS

Diets	Avg Food consumed /♂ fly (mg)	Avg Food consumed / ♀ fly (mg)
Corn diet	0.55	1.11
Ponni diet	0.57	1.12

Table 3.6 Average food consumed per fly in feeding cups

Feeding assay results shows that both male and female flies feed on Ponni diet. The values are very similar to each other.

3.4.2 FEEDING ASSAY-ERIOGLAUCINE DYE

The standard curve was plotted and the concentration of the dye in the sample was calculated.

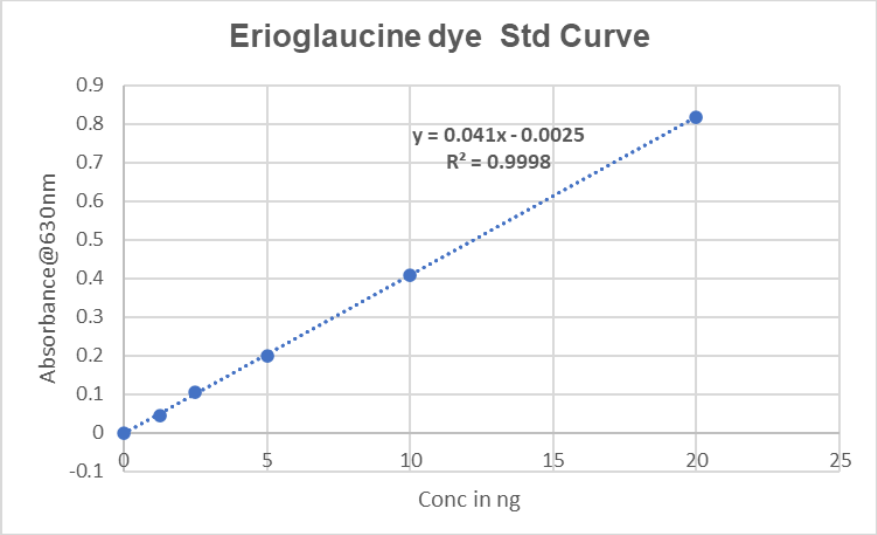

Figure 7: Standard curve of Eriogalucine dye

Diets	Avg Food consumed /♂ fly (ng)	Avg Food consumed / ♀ fly (ng)
Corn diet	0.12	0.72
Ponni diet	0.11	0.87

Table 3.7 Average food consumed per fly.

Feeding assay results shows that both male and female flies feed on Ponni diet. The values are very similar to each other.

The results from feeding assay, both methods, show that flies feed on Ponni diet at the same level as corn flour.

There are several factors that can affect fecundity. Food utilization in flies in stressful conditions could be another parameter that is affecting the egg laying capability. To check this aspect, flies that were stressed during development were taken to see any effects.

3.5 EGG LAYING IN CONTROL AND EARLY LIFE STARVED (ELS) FLIES FED ON RICE DIETS

On analyzing the fecundity of Control and ELS flies in each of the diets, control flies laid more eggs in corn however ELS flies laid more eggs in the rice media.

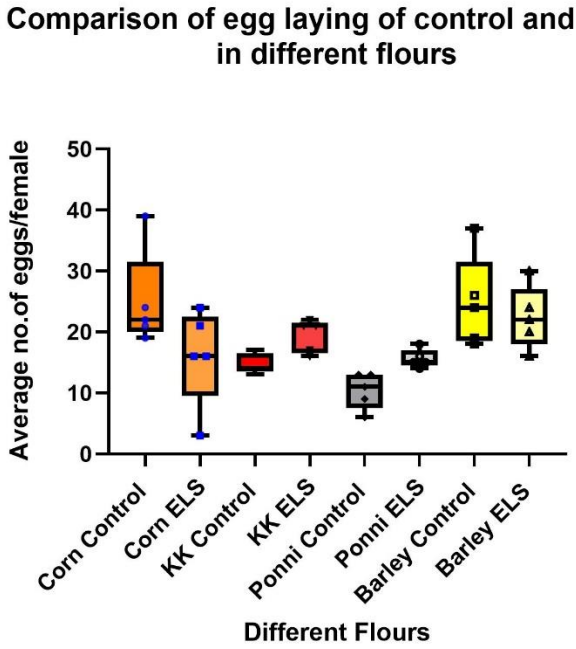

Figure 8: Comparison of egg laying in different flours; n= 5sets; 2 sets had 6♀,3♂, 3 sets had 3♀,2♂

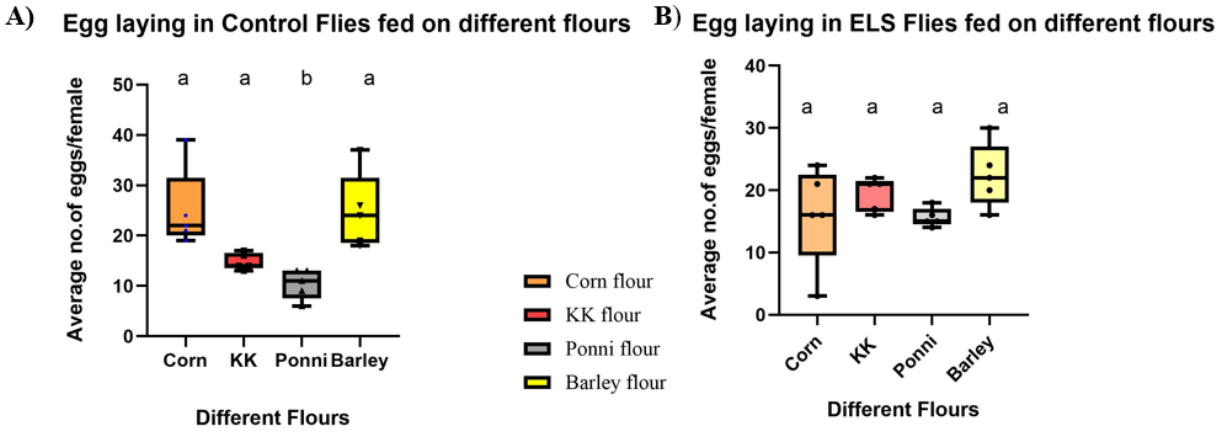

Figure 9: A) Egg laying of control flies fed on different diet B) Egg laying of ELS flies on different diets. One way ANOVA was performed. Letters above data indicate statistical differences between data sets by a post-hoc Tukey analysis.

It was very interesting to observe that the Early Life Starved (ELS) flies did better on the rice diets when compared to control flies.The flies that are starved early in development were able to utilise the nutrients from rice diet better.

Overall, egg laying in Karungkuruvai rice fed flies was comparable to control (corn) and barley fed flies.There was a decrease Ponni diet-fed flies. Further analysis needs to be done to check the role of any inhibitors or changes that occur at a molecular level affecting the expression of genes involved in the pathways related to nutrition and egg laying.

4.0 CONCLUSION

- From the various interactions with farmers, healers, and Siddha physicians, there is not much known about the nutritional and health benefits of Karungkuruvai rice. This rice is marketed as any black rice with a lot of nutritional benefits. This seems to be just a marketing way of propagating traditional rice. The rice might be nutritionally better than white rice but just being traditional does not imply it is one of the best rice available.

- Fecundity assay in *Drosophila melanogaster* model system is a good read-out to assess the nutritional aspect of the diet.

- On comparing flies fed on the Karungkuruvai rice diet and Ponni rice diet, egg laying was comparable to the control diet for both the short-term and long-term feeding not for the Ponni diet though.

- Further research needs to be done to understand the underlying biological mechanisms through which egg laying is reduced in Ponni rice, though from a macronutrient perspective, all the diets were similar.

- Overall, in terms of fecundity as a life history trait, Karungkuruvai rice is a better diet compared to the Ponni rice diet. Further analysis of the effects of rice diets on other life-history traits like life span, ability to deal with stress, etc. should be done to conclude which diet is a better diet for the fly system.

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APPENDIX

FECUNDITY ASSAY (Short Term Feeding)

Experiment Dates	Jan 27th - 30th 2023	Mar12th-15th 2023	Apr 11th-14th 2023	Female
Time	24hrs			3 Male
Days in life cycle	6-9			

Count of Eggs	Corn Flour		
	V1	V2	V3
Day 1	548	524	536
Day 2	488	511	496
Day 3	418	464	475
Total	1454	1499	1507

No. of Females	V1	V2	V3
Day 1	18	18	18
Day 2	18	18	18
Day 3	18	16	17

No.of Eggs/Female Day 1	30	29	30
No.of Eggs/Female Day 2	27	28	28
No.of Eggs/Female Day 3	23	29	28
Average	27	29	28

Avg. Eggs on Corn Flour /Day	28

Count of Eggs	Barley Flour		
	V1	V2	V3
Day 1	436	406	528
Day 2	384	390	470
Day 3	375	352	396
Total	1195	1148	1394

No. of Females	V1	V2	V3
Day 1	18	18	18
Day 2	17	17	18
Day 3	16	15	16

No.of Eggs/Female Day 1	24	23	29
No.of Eggs/Female Day 2	23	23	26
No.of Eggs/Female Day 3	23	23	25
Average	23	23	27

Avg. Eggs on Barley	24

Count of Eggs	KKFlour		
	V1	V2	V3
Day 1	547	434	455
Day 2	481	389	377
Day 3	413	354	373
Total	1441	1177	1205

No. of Females	V1	V2	V3
Day 1	18	18	18
Day 2	18	17	18
Day 3	18	16	15

No.of Eggs/Female Day 1	30	24	25
No.of Eggs/Female Day 2	27	23	21
No.of Eggs/Female Day 3	23	22	25
Average	27	23	24

Avg. Eggs on KK Flour /Day	24

Count of Eggs	Ponni Flour		
	V1	V2	V3
Day 1	337	460	419
Day 2	309	365	389
Day 3	228	282	318
Total	874	1107	1126

No. of Females	V1	V2	V3
Day 1	18	18	18
Day 2	17	18	18
Day 3	16	18	16

No.of Eggs/Female Day 1	19	26	23
No.of Eggs/Female Day 2	18	20	22
No.of Eggs/Female Day 3	14	16	20
Average	17	21	22

Avg. Eggs on Ponni Flour /Day	20

FECUNDITY ASSAY (Long Term Feeding)

Experiment Dates	Feb 2nd-5th 2023	Mar26th-29th 2023	6 Female
Time	24hrs		3 Male
Days in life cycle	6-9		

Count of Eggs	Corn Flour		
	V1	V2	V3
Day 1	346	268	331
Day 2	283	239	296
Day 3	229	194	199
Total	858	701	826

No. of Females	V1	V2	V3
Day 1	12	12	12
Day 2	12	10	12
Day 3	12	11	12

No.of Eggs/Female Day 1	29	22	28
No.of Eggs/Female Day 2	24	24	25
No.of Eggs/Female Day 3	19	18	17
Average	24	21	23

Avg. Eggs on Corn Flour	23

Count of Eggs	Barley Flour		
	V1	V2	V3
Day 1	333	262	355
Day 2	312	209	246
Day 3	253	162	203
Total	898	633	804

No. of Females	V1	V2	V3
Day 1	12	12	12
Day 2	12	12	12
Day 3	12	12	12

No.of Eggs/Female Day 1	28	22	30
No.of Eggs/Female Day 2	26	17	21
No.of Eggs/Female Day 3	21	14	17
Average	25	18	22

Avg. Eggs on Barley	22

Count of Eggs	KKFlour		
	V1	V2	V3
Day 1	351	362	329
Day 2	316	347	318
Day 3	306	280	263
Total	973	989	910

No. of Females	V1	V2	V3
Day 1	12	12	12
Day 2	11	10	12
Day 3	11	9	11

No.of Eggs/Female Day 1	29	30	27
No.of Eggs/Female Day 2	29	35	27
No.of Eggs/Female Day 3	28	31	24
Average	29	32	26

Avg. Eggs on KK Flour /Day	29

Count of Eggs	Ponni Flour		
	V1	V2	V3
Day 1	202	172	141
Day 2	180	122	112
Day 3	114	126	84
Total	496	420	337

No. of Females	V1	V2	V3
Day 1	12	12	12
Day 2	12	12	10
Day 3	12	12	10

No.of Eggs/Female Day 1	17	14	12
No.of Eggs/Female Day 2	15	10	11
No.of Eggs/Female Day 3	10	11	8
Average	14	12	10

Avg. Eggs on Ponni Flour /Day	12